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AWARD NUMBER DAMD17-97-1-7266

TITLE: The Role of NF-kB in Normal and Transformed Mammary Epithelium

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REPORT DATE: August 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE August 1998	3. REPORT TYPE AND DATES COVERED Annual (1 Aug 97 - 31 Jul 98)
4. TITLE AND SUBTITLE The Role of NF- κ B in Normal and Transformed Mammary Epithelium			5. FUNDING NUMBERS DAMD17-97-1-7266
6. AUTHOR(S) Yull, Fiona			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Vanderbilt University Medical Center Nashville, Tennessee 37232			8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research And Materiel Command ATTN: MCMR-RMI-S 504 Scott Street Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER
11. SUPPLEMENTARY NOTES <div style="text-align: right; font-size: 2em; font-weight: bold;">19990603 080</div>			
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited			12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 words) <p>Breast cancer is a major cause of cancer mortality for women in the United States and represents the highest rate of new cancer cases for women. A knowledge of the molecular signalling pathways which when perturbed result in mammary cell transformation will provide us with vital information in the fight against this disease. The NF-κB family of transcription factors are a major component of signal transduction pathways regulating cell proliferation, differentiation and death. We are attempting to determine the role they play in the development of normal and transformed mammary epithelium. Our hypothesis is that nuclear expression of specific NF-κB factors is necessary for the proper differentiation and maturation of mammary epithelium and that alteration in the expression of κB factors can effect transformation of mammary epithelium. We have created lines of transgenic mice which perturb NF-κB levels by overexpression of the family member already suspected to be involved in carcinogenesis (NFκB2) and of a dominant negative inhibitor of NF-κB activity (IκBα-ΔN) in the mammary gland. Preliminary characterization of expression patterns and phenotypes are presented.</p>			
14. SUBJECT TERMS Breast Cancer NF- κ B, transgenics			15. NUMBER OF PAGES 18
			16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

FOREWORD

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Fiona E. Hall 8.24.98
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INTRODUCTION

Breast cancer is a major cause of cancer mortality for women in the United States and represents the highest rate of new cancer cases for women (1). Enormous scientific and medical resources have been invested over the past two decades aimed at preventing and curing these conditions. While many significant advances have been made at both the clinical and molecular levels towards an understanding and eradication of the cancer phenotype, the remaining high morbidity and mortality rates suggest that increased knowledge about mammary carcinogenesis is vital. This proposal has two major goals. We plan to characterize the, as of yet, unexplored role of the transcription factor complex, nuclear factor- κ B, NF- κ B) in normal mammary development. We also plan to study the interesting correlation between the presence of an unusual isoform of NF- κ B, NF κ B2, and breast adenocarcinomas. To address these questions we are generating novel transgenic models.

NF- κ B is classically considered a transcription factor of lymphoid cells. However, its presence in every cell type suggests an involvement in fundamental processes only recently being realized. Results from the targeted disruption of NF- κ B factors in murine lines show that κ B components have a role in cellular proliferation, differentiation, migration, and programmed cell death (5).

The epidemiology of cancers in humans and in animal models recognizes that multiple genetic events, often characterized by many distinct changes in molecular and phenotypic markers can be ascertained (6). During the development of mammary tumors, these stages can be used to define further molecular points of interest at which clinical intervention might be directed: normal, unaffected tissue \rightarrow hyperplasia \rightarrow induction of angiogenesis \rightarrow neoplasia \rightarrow metastasis. The rationale for NF- κ B involvement stems from studies in which NF- κ B has been shown to control the expression of growth related gene products (c-myc, p53, Gro proteins, PDGF-receptor β) and genes coding for matrix metalloproteinases and adhesion molecules (urokinase, type IV collagenase, ICAM-1, VCAM-1, etc.)(5). These observations suggest that dysregulation of an NF- κ B complex could easily result in altered regulation of these gene products leading to unscheduled growth, misregulated differentiation, excess angiogenesis or invasiveness.

The Role of NF- κ B in vivo

While a large number of oncogenic proteins are mediators of signal transduction, the Rel/NF- κ B family of transcription factors represents a distinct subset of nuclear transactivating factors whose activity is inducible via control of its nuclear localization. These enhancer binding proteins are sequestered in the cytoplasm by inhibitory molecules, termed I κ Bs. Upon stimulation the I κ B is degraded, and the κ B factors are released to enter the nucleus and associate with their cognate DNA binding sites initiating gene transcription. Recent observations suggest that these factors are involved not only in lymphoid cell differentiation, but also in regulation of the cell cycle in many cell types, in regulating programmed cell death, and in embryonic patterning of limb and neuronal tissues. Given their involvement in aspects of cell differentiation, proliferation and apoptotic cell death, we feel that a thorough characterization of the role of the Rel/NF- κ B family members in the development of the mammary gland would provide key information to improve our understanding of both normal and pathogenic morphogenesis.

NF- κ B

The Rel/NF- κ B family of transcriptional transactivators is an ubiquitous multiprotein complex specialized for rapid response of the cell to a wide variety of both normal and pathogenic agents (5). The product of the protooncogene *c-rel*, cRel, and the nuclear factor- κ B, NF- κ B, act as transcription factors by binding to a decameric sequence motif found in the promoters and introns of several genes; including those of the immunoglobulin κ light chain, the human immunodeficiency virus long terminal repeats, numerous adhesion molecules, cytokine and growth factor receptors. The molecular, biochemical and physiological nature of the Rel/NF- κ B complex has been well reviewed (5,7,8,9). Authentic NF- κ B is a heterodimer consisting of a 50 kD polypeptide (NF κ B1=p50) (10) and a 65 kD polypeptide (RelA=p65)(11) whose primary sequence reveals that the amino-terminal halves of both p50 and p65 are highly homologous to that of the *rel* protein (defining the Rel homology domain, RHD). The RHD encompasses the DNA-binding, dimerization, I κ B-binding, basal transcription factor binding, and nuclear localizing domains for these proteins. The carboxy terminal domains of RelA(p65) and cRel contain strong transcriptional transactivation regions (12). In contrast, p50 homodimers bind DNA but are thought to block transcription (13). In vertebrates, other genes also encode factors which participate in the NF- κ B complex and bind to κ B enhancer elements: NF κ B2 (p52 and its precursor p100)(14-17), relb (RelB), and v-rel. Two rel-related homologs, *dorsal* and *dif* have been identified in *Drosophila* where they aid in determining the developmental fate of cells along the dorsal-ventral axis during embryogenesis (18,19).

I κ B

I κ B was first described as a cytoplasmic protein which inhibits the DNA binding activity of the transcription factor NF- κ B heterodimeric complex (20,21). In the cytosol of all cell types examined, I κ B proteins form a complex with heterodimeric NF- κ B, and can be inactivated following stimulation of cells with a wide variety of distinct agents including many cytokines (IL-1, IL-2, IL-8, etc.), growth factors (TNF- α , PDGF, etc.), lipopolysaccharide, and ultraviolet light. Like their binding partners NF- κ B, the I κ Bs are encoded by a small multigene family (5,9). These genes include I κ B- α , I κ B- β , I κ B- γ , and BCL-3. Following stimulation of cells, the I κ B- α protein is phosphorylated on serine residues in the amino-terminus targeting it for destruction by the ubiquitination/proteasome (26S) degradation pathway, allowing the translocation of NF- κ B to the nucleus (22,23,24). Our studies and others reveal that a mutation in the I κ B- α which removes these serine residues creates an I κ B- α protein (termed I κ BAN) which behaves as a transdominant negative inhibitor to the NF- κ B complex. Thus, because the serine target for the growth factor-induced phosphorylation is missing, the I κ BAN can efficiently bind NF- κ B in the cytoplasm but cannot be inactivated or degraded in response to physiological or pharmacological signals (22,23,24). This has provided an effective means by which to "capture" NF- κ B in a cytoplasmic-bound state, thereby preventing its transactivating capabilities. We are currently using this mutant in transgenic mouse lines in which the expression of I κ BAN has been directed to specific tissues to examine the role of NF- κ B factors during development.

The Role of NF- κ B in Mammary Development and Breast Adenocarcinoma

Preliminary evidence from this laboratory described below and work from Dejardin *et al.*, describes the overexpression of the NF κ B2 gene product, p100/p52 in adenocarcinomas of the breast (25). Analysis of human breast cancer primary tumor as well as breast cancer cell lines reveals a high

percentage (75%) which display overexpression of the NF κ B2 precursor protein, p100. NF κ B2 was originally identified via its involvement in a lymphoma-associated chromosomal translocation and is expressed as a 3.2 kilobase mRNA encoding a 100 kDa protein (14,15). Following proteolytic cleavage to the mature p52 kDa protein, NF κ B2-p52 can heterodimerize with all known κ B factors and activate the transcription of genes containing κ B-enhancer elements. The NF κ B2-p100 precursor has activity on its own; p100 behaves as an I κ B molecule by sequestering NF- κ B complexes in the cytoplasm (16). In one lymphoid-tumor case reported, overexpressed NF κ B2 proteins resulted in abnormal transcriptional regulatory properties (17) suggesting that alteration in NF κ B2-regulated gene expression may be causal for transformation. These results suggest that overexpression of either the precursor molecular, p100, or its processed fragment, p52, might alter the normal cellular NF- κ B complexes and dysregulate cellular homeostasis.

Expression of transgenes in murine mammary gland

The major whey protein of ruminants is B-lactoglobulin (BLG). Despite the fact that rodents do not have an endogenous BLG gene transgenic mice carrying BLG constructs express specifically and abundantly in the murine mammary gland in an appropriate temporal pattern (26,27). Transgenes using 5' and 3' BLG flanking sequences can target expression of heterologous genes in an appropriate developmental pattern to the mammary (28,29,30). Our expertise in mammary-directed transgenics coupled with the ability to examine physiologically relevant NF- κ B targets will provide necessary insight into mammary gland development and, hopefully, intuition into the process of mammary carcinogenesis.

BODY

Results: mammary RO1 (1) Transgenic analysis of BLG-NF κ B2 mice (BLG-inhibitor =BIN).

In order to express the individual κ B factors of interest, a mammary gland specific transgenic promoter (β -lactoglobulin, BLG) characterized by Dr. John Clark's laboratory in the Edinburgh Research Station at the Roslin Institute was used. The 4.2 kb promoter of the major milk whey protein of sheep, β -lactoglobulin, has been demonstrated to drive high level, mammary gland-specific expression of transgenes (3,4,27). The BLG promoter directs tissue-restricted expression in 10 days post-coitem mammary gland. The level of expression increases throughout pregnancy, reaching a peak during lactation. Using Dr. Clark's pBJ41 construct, a transgene bearing the human NF κ B2 cDNA has been constructed by standard molecular cloning techniques (Figure 1A). The plasmid is called pBLG-NF κ B2. In our original proposal we stated that should the efficiency of generating expressing lines prove to be low we would "rescue" gene expression by coinjection of a genomic BLG construct with the hybrid BLG/transgene construct which has been shown to significantly increase the efficiency of obtaining expressing transgenic lines (28,29). We decided to employ this strategy from the start. The purified transgene fragment was coinjected together with the BLG genomic fragment by the Vanderbilt Cancer Center Transgenic Core. Four founder animals of BLG-NF κ B2/p100 (BIN) were identified from 31 pups. Southern analysis of tail DNA from founder animals confirms the presence of the transgene (Figure 1B). Line #6 is probably single copy but its presence is confirmed by PCR analysis (Figure 1C). The PCR primer pairs are designed such that an endogenous HPRT product acts as a control for successful PCR reactions. Primers which amplify a fragment from the BLG 5' flanking region identify transgenics. The transgene has transmitted to subsequent generations in all lines.

G1 transgenic offspring and control animals are being analyzed for expression of the transgene by northern blot analysis for (1) tissue specificity, (2) expression during mammary gland development at specific timepoints throughout pregnancy, lactation and regression (virgin, 10.5 and 16.5 dpc, 1.5 and 9.5 lactating and 1, 3, 5 and 40 days post forced wean at day 10 of lactation). At each stage one gland is removed for histological processing, one is processed in TriZol reagent for RNA and protein extraction, one is flash frozen for isolation of cytoplasmic and nuclear proteins for EMSA, and one gland is preserved for whole mount gland preparation. The particular gland collected for each type of analysis remains constant.

Initial characterization of expression is being carried out by Northern blot analysis. Figure 2A shows expression of the transgene in mammary samples from lines BIN#6, #10, #16, and #18 at 16.5

dpc, 1.5 lactating and 9.5 lactating. To detect NF κ B2 mRNA, probes of human origin are used that do not cross-react with the endogenous murine NF κ B2. In these preliminary analyses we have been unable to detect transcripts from the Bin #6 line. However, this line is proving difficult to propagate so at this stage we are continuing characterization. The other three lines all appear to express the transgene at the predicted stages of mammary development. A consistent increase in expression from 16.5 dpc through early to late lactation would be predicted, however, these early results indicate variation between animals. Figure 2B is a preliminary analysis of tissue specificity in line BIN#10. While the expression in the mammary is difficult to visualize in this example it is, in fact, the same RNA as that shown in Figure 2A. We are intrigued by the relatively extremely high levels of expression in the brain. Analysis of other lines will determine whether this is ectopic expression of the transgene in this line only or is a consistent observation.

Whole mount analysis of gland morphology will be performed on each timepoint collected mammary gland. Shown are preliminary examples of glands harvested at 10.5 dpc (Figure 3). We are interested in differences in ductal width, length, degree of branching and number of terminal end buds. There appear to be striking differences in the degree of development, particularly apparent in the comparison between BIN#16 and the non-transgenic control. However, at this time, we have only collected a minimal number of examples and have not characterised "between animal" variation.

Slide sections are initially being stained with hematoxylin and counterstained with eosin (H&E) to analyze morphological differences. Shown are the initial examples of mammary glands collected at 16.5 dpc (Figure 4). A general observation is a decrease in alveolar proliferation in specific regions leading to the appearance of "dense" cells. In glands collected at later stages we observe a reduction in milk production. In the future, sections will be resources for analyses using other biochemical, molecular, and immunohistochemical markers. Such immunohistochemical analysis will confirm expression of the NF κ B2 transgenic protein and determine whether the product is present as p100 or the processed p52 form.

Our very early results suggest that overexpression of NF κ B2 may have morphological consequences during mammary gland development. BIN lines appear to display a decrease in the proliferation of ductal epithelial cells at early stages of pregnancy shortly after the beginning of transgene expression (this decrease still needs to be fully characterized). We have noted a decreased number of alveoli, with reduced lipid content at early stages (16.5 dpc).

Analysis of BLG-I κ B- α Δ N transgenic mice (mammary - α Δ N =MAN).

Similar to the design for the BLG-NF κ B2 transgene, the avian I κ B- α Δ N was subcloned into the pBJ41 construct. Using Dr. Clark's pBJ41 construct, a transgene bearing the avian I κ B- α Δ N has been constructed by standard molecular cloning techniques. The plasmid is called pBLG- Δ N. The avian I κ B- α Δ N has been used successfully in this laboratory to generate transgenic lineages expressing I κ B- α Δ N specifically in B cells in order to inhibit NF- κ B activity (30). The purified transgene fragment was cojoined together with the BLG genomic fragment by the Vanderbilt Cancer Center Transgenic Core. Four positive founder animals of BLG-I κ B- α Δ N(MAN) were identified from 29 pups. Transgenic founders were confirmed by Southern blot of tail DNA and subsequent progeny are identified by PCR-based analysis as shown above. Transgenic lines are being propagated by mating with F1 mice. To characterize expression patterns, female MAN transgenics and control animals have been placed into matings to generate specific timepoints in duplicate throughout pregnancy, lactation and regression (virgin, 10.5 and 16.5 dpc, 1.5 and 9.5 lactating and 1, 3, 5 and 40 days post forced wean at day 10 of lactation). As mentioned above, each mouse has an array of tissues collected for several purposes. Full tissue resection sets are harvested for analysis of transgene expression. One mammary gland is processed into TriZol reagent for RNA and protein extraction. One mammary gland is flash frozen for future cytoplasmic and nuclear protein extraction for use in EMSA. One mammary gland is fixed and processed into paraffin sections for immunohistochemistry or *in situ* hybridization analyses and one gland is preserved for whole mount gland morphology.

Initial characterization of expression is being carried out by Northern blot analysis. Figure 5A shows expression of the transgene in mammary samples from lines MAN#8, #13, #18, and #19 at 16.5 dpc, 1.5 and 9.5 lactating. We have identified at least one expressing animal from each line. It is interesting to note that the 9.5 lactating MAN#8 sample, a stage at which one would predict maximal expression, is blank. The genotype of this animal was confirmed post-sacrifice by Southern analysis and the fidelity of the RNA confirmed. This effect remains to be further characterized. Figure 5B is a northern blot showing a representative time course of expression (line MAN #13). Similar analyses of other lines

and analyses of tissue specificity are underway. We are in the process of gathering probes for whey acidic protein (WAP), β -casein, *Msx-1*, cyclin-D, and murine BRCA-1 and -2 to examine the potential affect of transgene expression on these markers of mammary proliferation and differentiation.

Given the mosaic nature of transgene expression in some tissues, sectioned specimens will be analyzed directly for transgene expression. This is somewhat problematic for the I κ B- α ΔN protein - our available COOH-terminal antisera does work well in immunohistochemistry. We are currently looking into the possibility of producing a new COOH-terminal antibody to an alternative domain.

Whole mount analysis of gland morphology will be performed on each timepoint collected mammary gland. Shown are preliminary examples of glands harvested at 10.5 dpc (Figure 6). Whilst the MAN#13 gland appears to have a striking reduction in the degree of development, we are still uncertain of the degree of natural variation between animals.

As is the case for the BIN transgenic lines our early data leads us to suspect a proliferation defect. Therefore, we are using a ribonuclease protection assay [RiboQuant, produced by Pharmingen] to begin to investigate the levels of cyclins in the mammary glands. Our first set of results are shown in Figure 7. We are intrigued to note the reduction in Cyclin D1 RNA. Animals deficient in cyclin D1 have reduced mammary development (31).

Mammary epithelial transformation and neoplastic progression, like most cancers, involves cooperative changes in more than one oncogenic or tumor-suppressor pathway. It is our intention to cross selected BIN and MAN lines with mice expressing the mouse mammary tumor virus (MMTV) driven-activated *neu* transgene, which reproducibly develop clonal mammary tumors, to determine whether transgene expression can either inhibit or cooperate in producing mammary tumorigenesis. Furthermore, BLG-NF κ B2/p100 expressing mice will be interbred with mice expressing the most common p53 mutation (Arg¹⁷²-His) in human breast cancers to explore the ability of NF κ B2 to act as a dominant oncogene in the absence of the well characterized tumor suppressor, p53. All combined, these interbreedings will explore the cooperative nature between these NF- κ B components and known mammary carcinogenic signal transduction and genetic pathways in the hopes of better understanding the mechanistic interactions of the oncogenic pathways in human mammary tumorigenesis. The lines of mice described above exist on an FVB strain background whereas our lines are on a B6/D2 background. In order to avoid the potential difficulties of interpreting data on a background consisting of a genetic mix of three strains we have begun to backcross our lines onto the FVB strain.

In our original proposal we intended to analyze pBLG-NF κ B2 mice for age related effects on mammary physiology by comparison between transgenics on a continuous mating schedule versus virgin females to compare the effects of pregnancy versus non-pregnant cycling of mammary tissue in the presence of overexpressed NF κ B2. Ms. Brantley, a pre-doctoral student in this lab, has been trained in pituitary isografts in which the entire pituitary of a donor mouse is placed under the kidney capsule of the recipient mouse thus maintaining circulating hormone environment which mimics pregnancy. This strategy is being employed to simplify the task of maintaining constant transgene expression. The advantages of such a procedure are (a) that the pituitary isograft is functional three weeks after the graft, (b) it lasts the lifespan of the animal, and (c) can remove graft anytime by surgery or by cautery.

We intend to cross our transgenic lines with reporter lines generated in this lab designated HLL (32). The HIV-1 LTR has been extensively characterized as a κ B responsive promoter (33). These lines carry the κ B responsive human immunodeficiency virus-1 long terminal repeats, HIV-LTR, promoter fused to luciferase which enables direct analysis of the expression of an *in vivo* κ B reporter. This will provide an extremely valuable system which will allow us to determine whether the overexpression of NF κ B2 results in an active transcription factor or whether the full length protein (p100) is behaving as an inhibitor of other NF- κ B complexes and thus, preventing transcriptional activation.

CONCLUSIONS

Present data suggests that we have achieved our goal of generating four distinct expressing lines of each transgene type. Analysis of these lines is at an extremely early stage. However, we are encouraged to be able to detect expression with ease in seven of the eight lines. Our initial characterization studies are already highlighting differences between transgenics and wild-type animals.

At this stage we are uncertain of the degree of natural variation between animals, particularly given that our transgenics are on a B6/D2 background ie. A mixture between C57Bl6 and DBA strains. We are already backcrossing our animals onto the FVB strain to alleviate any potential difficulties associated with such variation. Such backcrosses are already necessary for our proposed tumorigenesis studies.

We are extremely interested in determining whether the transgenes are expressed consistently across entire glands or whether some degree of mosaic expression may be present.

Our hypothesis is that the BIN lines will be found to have different levels of expression p100:p52 ratios thus altering the inhibitor:transactivator ratio within the cells. This would afford us the unique opportunity of dissecting the roles of these precursor:mature NF κ B2 proteins in mammary gland proliferation, function and regression. However, such studies are dependant on results from planned western and EMSA analyses.

In summary, in relation to the original statement of work, samples collected for technical objective 1 have been included in the results reported for award #DAMD17-97-1-7017. Tasks 2-4 of technical objective 2 have been completed and some inroads have been made into Tasks 5 and 6. Therefore, we are happy to be able to report progress well in line with the predicted schedule.

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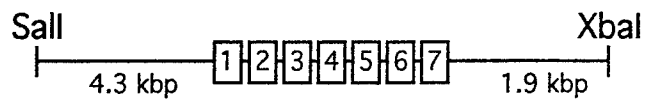
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Figure 1

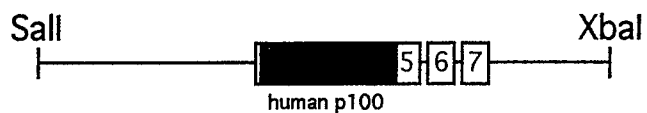
APPENDICES

β -lactoglobulin (BLG)
(pSSltgXS)

A

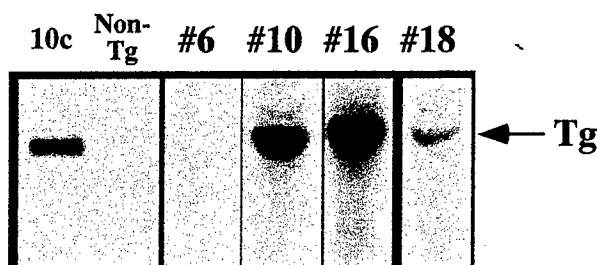


BLG-NFKB2-p100
(pBJ41-p100)



pBLG-NFKB2/p100

B



C

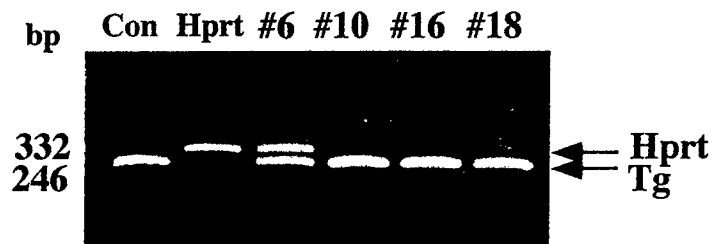


Figure 2

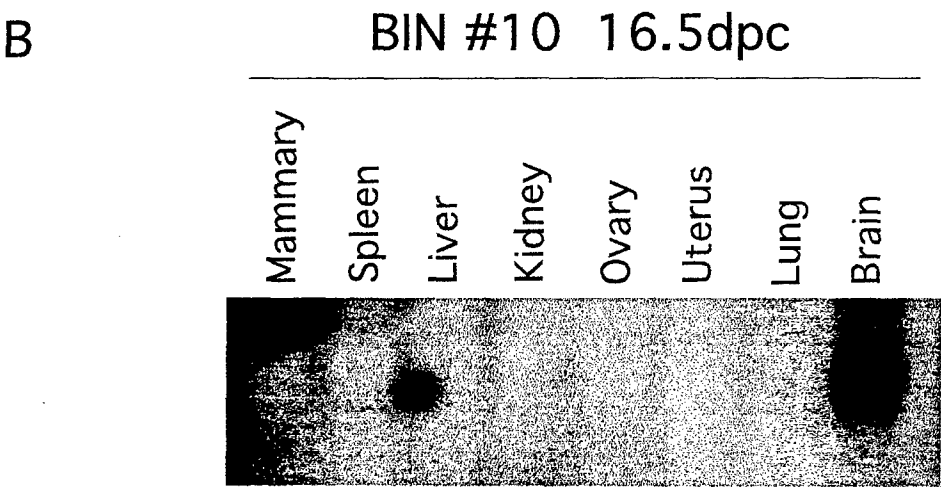
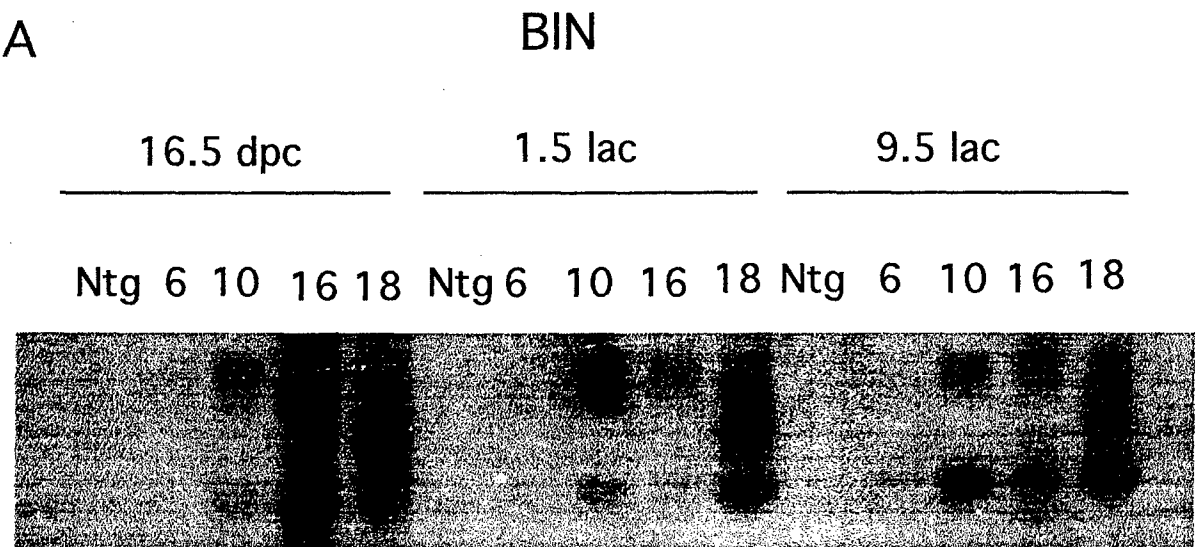
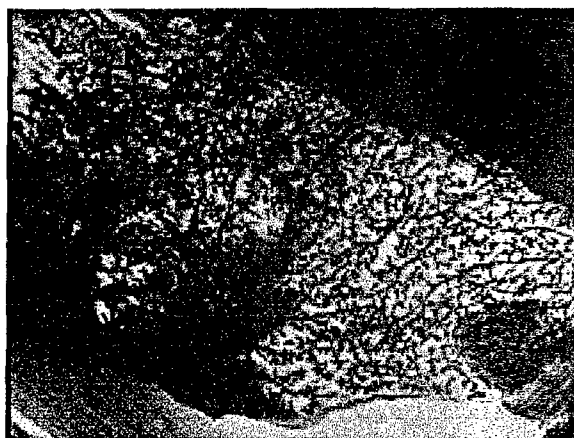


Figure 3

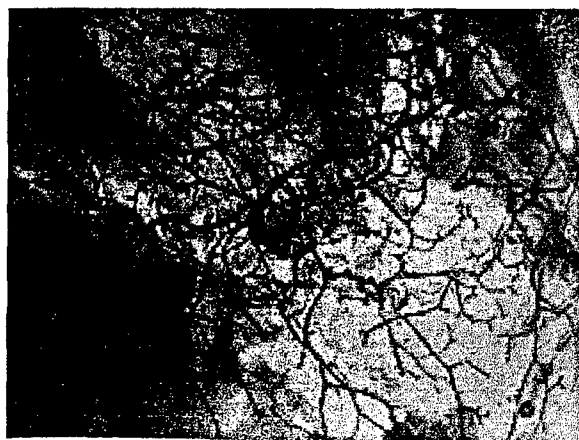
10.5 dpc



non-Tg



BIN 6.3.15



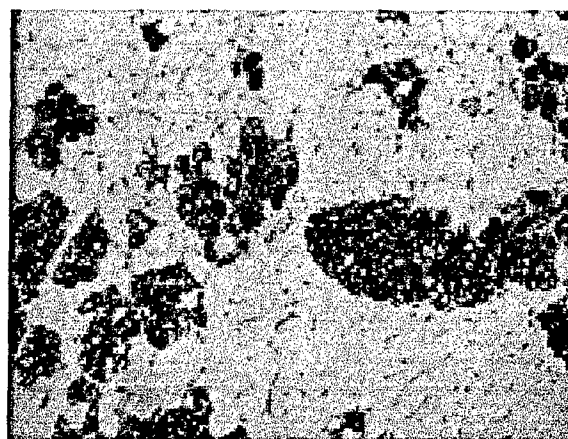
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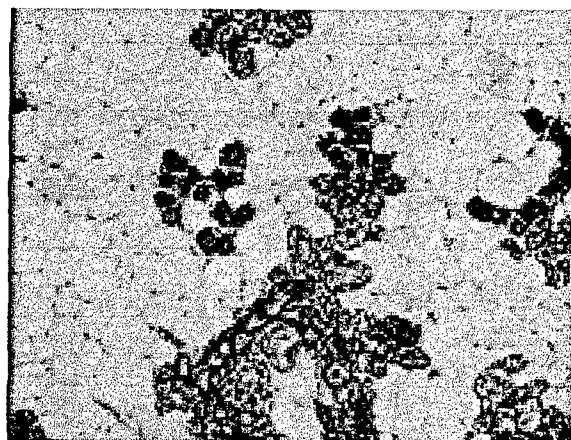
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Figure 4

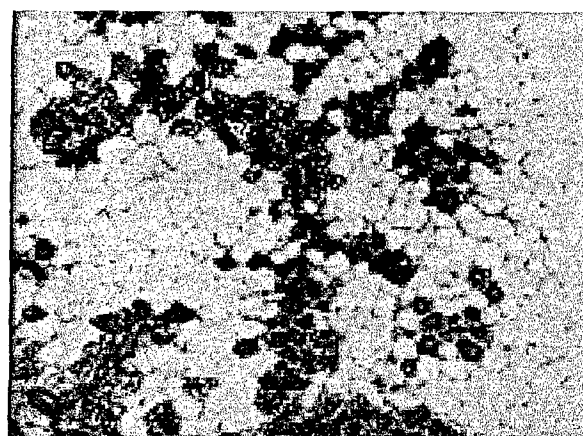
16.5 dpc



Non-Tg



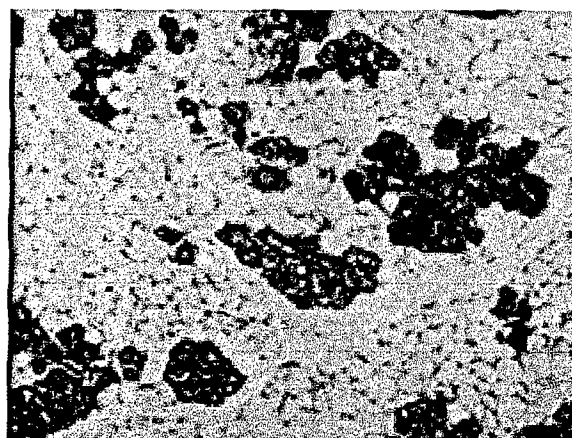
BIN 6.4



BIN 10.24



BIN 16.10



BIN 18.38

Figure 5

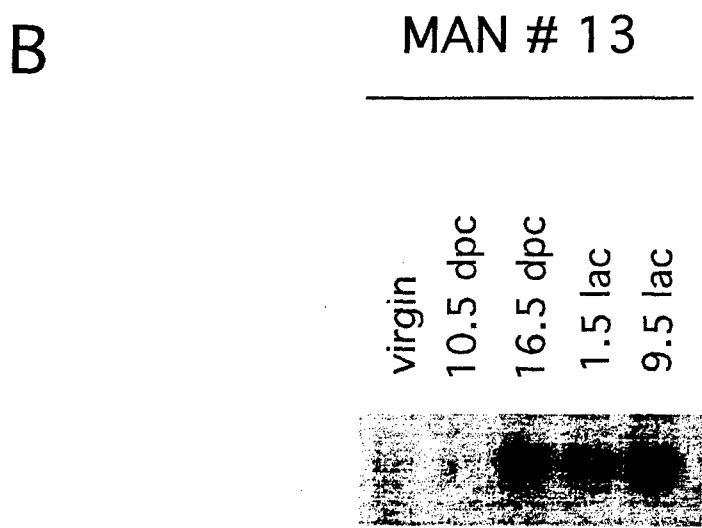
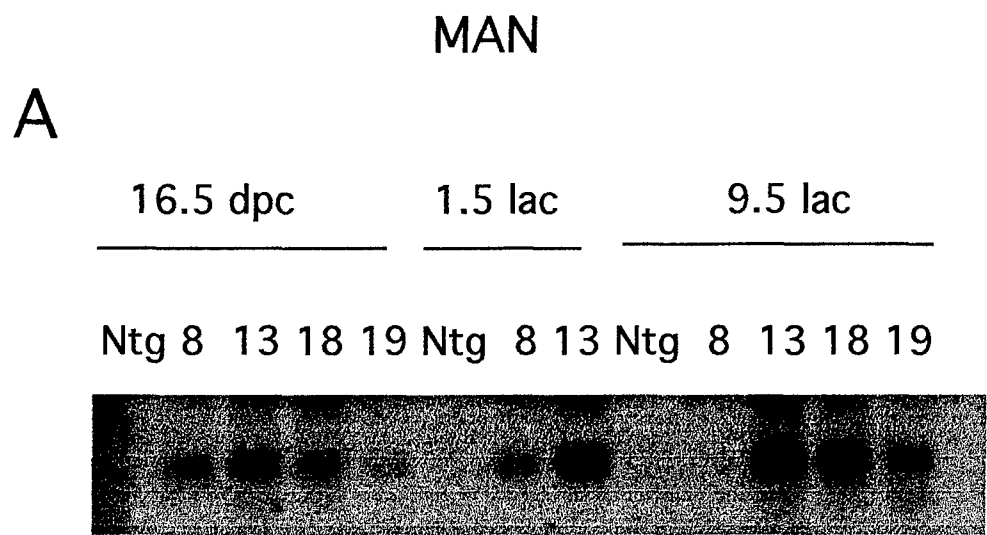


Figure 6

10.5 dpc



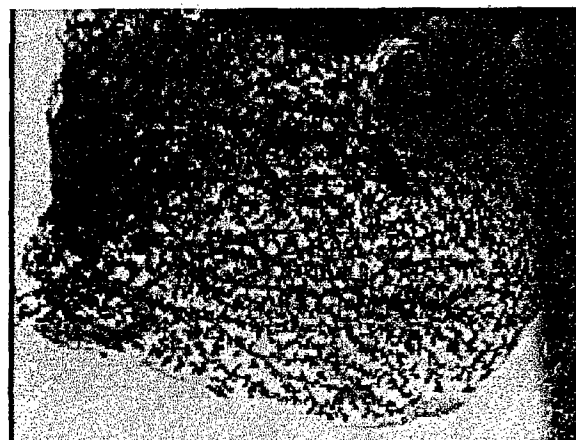
non-Tg



MAN 8.33



MAN 13.6



MAN 19.40

Figure 7

